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Date of Signature and Deposit: March 19, 2003

Nicholas J. Seay

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: James A. Thomson

Serial No.: 09/522,030

Filed: 03/09/2000

For: SERUM FREE CULTIVATION OF PRIMATE EMBRYONIC STEM CELLS

Date: March 14, 2003

Group Art Unit: 1632

Examiner: Joseph T. Woitach

File No.: 960296.96544

DECLARATION

Commissioner For Patents
Washington, DC 20231

Dear Sir:

I, James A. Thomson, on oath deposes and says that:

1) I am the inventor of the above-identified patent application and make this declaration in support of this patent application.

2) I understand that an issue being considered by the Examiner of this patent application is whether the effect of basic fibroblast growth factor (bFGF) on the culture of embryonic stem cells, as described in this patent application, is unique to bFGF or is an effect that could be achieved using other fibroblast growth factors. I believe that the effect is achieved by other fibroblast growth factors.

3) Attached to this declaration is a paper by Ornitz et al, a paper cited in this patent application. On page 15296 of that paper is a table that shows that all nine fibroblast growth factors then known act, in varying degrees of intensity, on the same set of receptors. This data suggest to me that any of these nine fibroblast growth factors would have the same

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biological effect on human embryonic stem cells, although some fibroblast growth factors might need to be applied at higher concentrations than others. FGF2 on the Ornitz table is bFGF, while FGF1 on the Ornitz table is acidic fibroblast growth factor (aFGF).

4) To further test my belief, I had experiments performed to test the effect of aFGF on human embryonic stem cell culture. Subcultures of human embryonic stem cells were made in my laboratory and were cultured in media containing commercial serum replacement and are of the following three conditions: (1) with aFGF, (2) with bFGF, or (3) with neither aFGF or bFGF. The cell cultures that resulted were photographed. A copy of that photograph is attached to this declaration. In the bottom set of pictures (labeled 20x) the ES cells cultured with aFGF and bFGF have identical morphology and appearance, typical of proliferating undifferentiated human embryonic stem cells, while the cell culture grown without either fibroblast growth factor has a morphology consistent with differentiation into other types of cells.

5) When my laboratory was experimenting with using serum replacement products to culture stem cells in serum-free media, we tried other factors as additives to the serum-free media using serum replacement products, but did not achieve success except with a fibroblast growth factor. Other factors did not, in our hands, prove successful. For example, we attempted to culture human embryonic stem cells with serum replacement medium and with leukemia inhibitory factor (LIF) since LIF was known to aid in the serum-free growth of mouse embryonic stem cells. In our hands, LIF did not make any difference in the growth of human embryonic stem cells.

6) I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

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States Code, and that such willful false statements may jeopardize the validity of the application, or any patent issuing thereon.

James A. Thomson

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Receptor Specificity of the Fibroblast Growth Factor Family*

(Received for publication, January 31, 1996, and in revised form, March 15, 1996)

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Fibroblast growth factors (FGFs) are essential molecules for mammalian development. The nine known FGF ligands and the four signaling FGF receptors (and their alternatively spliced variants) are expressed in specific spatial and temporal patterns. The activity of this signaling pathway is regulated by ligand binding specificity, heparan sulfate proteoglycans, and the differential signaling capacity of individual FGF receptors. To determine potentially relevant ligand-receptor pairs we have engineered mitogenically responsive cell lines expressing the major splice variants of all the known FGF receptors. We have assayed the mitogenic activity of the nine known FGF ligands on these cell lines. These studies demonstrate that FGF 1 is the only FGF that can activate all FGF receptor splice variants. Using FGF 1 as an internal standard we have determined the relative activity of all the other members of the FGF family. These data should serve as a biochemical foundation for determining developmental, physiological, and pathophysiological processes that involve FGF signaling pathways.

Fibroblast growth factor (FGF)[1] was identified as an activity that stimulates the proliferation of NIH3T3 cells (1). Currently, FGFs comprise a family of nine structurally related proteins (FGF 1–9). FGFs are expressed in specific spatial and temporal patterns and are involved in developmental processes, angiogenesis, wound healing, and tumorigenesis (2–5).

FGFs bind and activate high-affinity receptor tyrosine kinases. The cloning of FGF receptors (FGFRs) has identified four distinct genes (6–13). These receptors bind members of the FGF family with varying affinity (13–16), and alternative mRNA splicing leads to isoforms of these receptors which have unique ligand binding properties (15, 17, 18). An additional mechanism regulating FGF activity involves heparin or heparan sulfate proteoglycans, molecules which facilitate ligand-receptor interactions (12, 19, 20). FGFRs contain an extracellular ligand binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain. The extracellular domain determines ligand binding specificity and mediates ligand-induced receptor dimerization. Dimerization in turn results in one or more trans-phosphorylation events and the subsequent activation of the receptor (21).

The extracellular region of the FGFR contains three immunoglobulin-like (Ig-like) domains (6). Alternative mRNA splicing creates several forms of the FGF receptor which differ in their extracellular sequence and have unique ligand binding properties. One splicing event results in the skipping of exons encoding the amino-terminal Ig-like domain (domain I) resulting in a "short" two Ig-like domain form of the receptor (22). The ligand binding properties of the short (two Ig-like domain) and long (three Ig-like domain) FGFRs are similar.[2] However, the short form of the receptor may have a higher affinity for some FGFs than the long form (23). Changes in this alternative splicing pattern may correlate with the progression of several tumors toward malignancy (24, 25).

Another RNA splicing event utilizes one of two unique exons and results in three alternative versions of Ig-like domain III (referred to as domains IIIa, IIIb, IIIc) in FGFRs 1–3 (15, 18, 22). FGFRs containing alternatively spliced Ig-like domains IIIb ("b") and IIIc ("c") are expressed on the cell surface and bind FGF ligands. The IIIa ("a") splice form of the FGFR terminates within Ig-like domain III to yield a secreted extracellular FGF-binding protein with no known signaling capability (26). DNA encompassing the carboxyl-terminal half of Ig-like domain III in FGFRs 1, 2, and 3 have a remarkable conservation in both the number and arrangement of the intron/exon boundaries (18, 27–29). Expression of these receptor isoforms appears to be regulated in a tissue-specific manner with b exon expression restricted to epithelial lineages and c exon expression restricted to mesenchymal lineages (29–33). Unlike FGFRs 1–3, FGFR 4 is not alternatively spliced in this region (34).

Receptor binding specificity is an essential mechanism for regulating FGF activity. Specificity is determined by sequence differences among individual FGFRs, by alternative splicing, and by sequence differences among the nine FGF ligands. Knowledge of the paired interactions between the nine known FGFs and the major splice forms of the four known FGF receptors is essential to begin to discern the functions of FGFs during development. In this report we analyze the mitogenic activity of each FGF on BaF3 cell lines that express unique FGFRs. For the known FGFs, we have assembled the available published mitogenic activity data for BaF3 cells (35–37) and have filled in all the gaps for the remaining FGFs. We present a paired analysis of the activity of each FGF (1–9) on the b and c splice forms of the FGFRs 1–3 and on FGFR 4.

* This work was supported by Grant CA60673 from the National Institutes of Health and by the Beckman Young Investigator Program (to D. M. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 314-362-3908; Fax: 314-362-7058; E-mail: dornitz@pharmdec.wustl.edu.

[1] The abbreviations used are: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor.

[2] D. M. Ornitz, M. Goldfarb, and A. Chellaiah, unpublished data.

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TABLE I
Amino acid sequence identity between mouse and human FGFRs

FGFR	% Amino acid identity[a]		Species used[b]		Ref.	GenBank accession numbers[c]	
	Overall	Ig-II-TM[d]	"b"	"c"		Human	Mouse
1	98	99	M	M	12,15	X51803	U22324
2	96	97	H	M	16,40	X52832	M86441
3	92	98	M	M	14,18	M58051	M61881
4	88	92		M	41	X57205	X59927

[a] "c" splice forms were used in this analysis.
[b] M, mouse; H, human.
[c] Accession numbers for sequences used in this analysis.
[d] Including immunoglobulin-like domain II up to the transmembrane domain.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant FGF 1 was a gift from K. Thomas, Merck Inc.; human recombinant FGF 2 was a gift from J. Abraham, Scios Nova Inc.; mouse FGF 3 was a gift from M. Mathieu and C. Dickson and was purified from NIH3T3 cells expressing the murine FGF 3 cDNA (35). Human recombinant FGF 4 was a gift from Genetics Institute Inc.; human recombinant FGF 5 was purified from *Escherichia coli* as described (38). Human recombinant FGF 6 was purified from *E. coli* as described (39). Human recombinant FGF 7 was a gift from Amgen Inc.; mouse FGF 8b was purified from *E. coli* as described (36); mouse recombinant FGF 9 was obtained from Pepro Tech Inc. (37). The source of the FGFR cDNAs are: FGFR 1b (15), FGFR 1c (12), FGFR 2b (40), FGFR 2c (16), FGFR 3b (18), FGFR 3c (14), and FGFR 4 (41).

FGFR Expression Plasmids—Full-length cDNAs encoding the three immunoglobulin-like domain form of FGFRs 1b, 1c, 2b, and 2c were cloned into the MIRB expression vector as described in Refs. 18, 36, and 37. The FGFR 3 cDNAs were re-engineered to enhance signaling in BaF3 cells by constructing chimeric cDNAs encoding the extracellular region of FGFR 3 fused to the cDNA encoding the intracellular region and tyrosine kinase domain of FGFR 1. FGFR 31c has the extracellular region from FGFR 3c (14) and the transmembrane domain and tyrosine kinase domain from FGFR 1. The cDNA sequences were assembled using polymerase chain reaction-directed mutagenesis and polymerase chain reaction-mediated ligations. The amino acid sequence joining FGFR 3c (bold) to FGFR 1 (plain) is ~TDRAGrSLYLEIIYCTGA~; the transmembrane domain is underlined, the lower case "r" (Arg) is an exogenous amino acid added in the cloning. FGFR 31b has the extracellular region and transmembrane domain derived from FGFR 3b (18) and the tyrosine kinase domain derived from FGFR 1. The amino acid sequence joining FGFR 3b (bold) to FGFR 1 (plain) is ~VVAAVIL-CRLKSGTKK~, the transmembrane domain is underlined.

FR4/R1C and FR4Δ/R1C contain the extracellular domains of murine FGFR 4 and FGFR 4Δ, respectively, fused to the transmembrane and cytoplasmic domains of murine FGFR 1 (41). The amino acid sequence joining FGFR 4 (bold) to FGFR 1 (plain) is ~PEARYTDIIYCTGA~; the transmembrane domain is underlined. FGFR 4Δ is a mutated form of the FGFR 4 extracellular domain which lacks the Ig-like domain I. The mutation was designed to delete Ig-like domain I at the precise boundaries where other FGFR genes exclude Ig-like domain I by the alternative splicing of their mRNA. A dipeptide alanine-proline (ap) replaces Ig-like domain I in FGFR 4Δ yielding the fusion sequence ~ASEEMEQapDSLTSIS~. The sequence derived from the signal peptide exon is in bold type. FR4/R1C and FR4Δ/R1C cDNAs were inserted into the pMX expression vector (42).

Cell Culture and Mitogenic Assays—Suspension cultures of BaF3 cells (43, 44) were maintained in RPMI 1640 media (Life Technologies, Inc.) supplemented with 10% neonatal bovine serum, 10% conditioned media from WEHI-3 cells, L-glutamine, and penicillin-streptomycin/β-mercaptoethanol. To express FGFRs in BaF3 cells, 10^6 cells were electroporated with 20 μg of *Cla*I linearized MIRB-FGFR plasmids or *Sfi*I linearized pMX-FGFR 4 plasmids as described previously (14, 19, 42). Cells were selected in media containing 600 μg/ml G418 (Life Technologies, Inc.) and 10% WEHI conditioned media for 10–12 days. Pools of transfected cells were checked for mitogenic responsiveness to FGF 1 and then subcloned by limiting dilution. The clonal cell lines used in this study are designated FR1b-5 (FGFR 1b), FR1c-11 (FGFR 1c), FR2b-7 (FGFR 2b), FR2c-2 (FGFR 2c), FR31bQ-3 (FGFR 3b), FR31c-4 (FGFR 3c), FR4/R1C-1 (FGFR 4Δ).

For mitogenic assays, BaF3 cells expressing specific FGFRs were washed and resuspended in RPMI, 10% neonatal bovine serum, L-glutamine. 22,500 cells were plated per well in a 96-well assay plate in media containing 2 μg/ml heparin. FGFs, diluted in media containing 2 μg/ml heparin, were added to each well for a total volume of 200 μl/well.

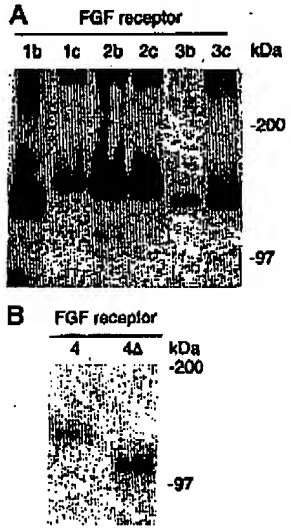

FIG. 1. FGF receptor expression in BaF3 cells. *A*, BaF3 cell lines expressing FGFRs 1–3 were cross-linked to ^{125}I-FGF 1 in the presence of heparin. *B*, BaF3 cells expressing FGFR 4-FGFR 1 chimeric molecules were visualized by Western blotting with antibodies directed against the carboxyl terminus of FGFR 1.

The cells were then incubated at 37 °C for 36–48 h. To each well, 1 μCi of [^{3}H]thymidine was added in 50 μl of media. Cells were harvested after 4–6 h by filtration through glass fiber paper. Incorporated [^{3}H]thymidine was counted on a Wallac β plate scintillation counter. Cell surface receptor cross-linking studies and Western blots were done essentially as described in Refs. 12 and 41.

RESULTS

FGFR-expressing Cell Lines—The BaF3 cell line has been used extensively to investigate the activity of a variety of receptor tyrosine kinases (19, 45–48). This cell line is dependent on interleukin 3 for growth. This dependence upon interleukin 3 can be replaced by ligands for receptor tyrosine kinases if the appropriate receptor tyrosine kinase is expressed in the BaF3 cell (19, 45). Wild-type BaF3 cells do not express FGFRs (14, 19, 41). However, BaF3 cells transfected to express FGFRs can be propagated in the presence of FGF (19, 41). To determine the relative mitogenic activity of FGFs 1–9 on different FGFRs, BaF3 cell lines have been engineered to express each of the three Ig-like domain, b and c splice forms, of FGFRs 1–3 or the two Ig-like domain forms of FGFR 4. The FGFRs used have been derived from either mouse or human cDNA clones. In the region encompassing Ig-like domain II to the transmembrane domain (the region thought to determine ligand binding specificity) there is a high degree of sequence conservation between these two species (92–99%) (Table I). It is therefore unlikely that species differences influence the ligand specificity of these

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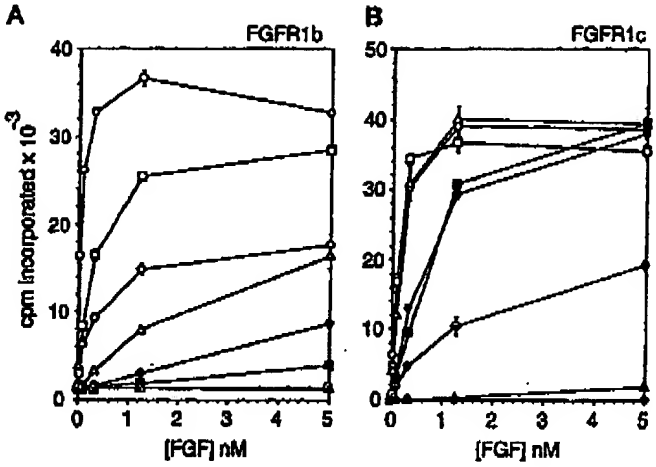

FIG. 2. BaF3 cell mitogenic assays-FGFR 1. *A*, FGFR 1b expressing cells; *B*, FGFR 1c expressing cells. *C*, the symbols (○, FGF 1; □, FGF 2; ◇, FGF 3; △, FGF 4; ●, FGF 5; ■, FGF 6; ◆, FGF 7; ▲, FGF 8; ⊖, FGF 9) are used to represent the nine FGFs in the mitogenic assays shown in Figs. 2–5.

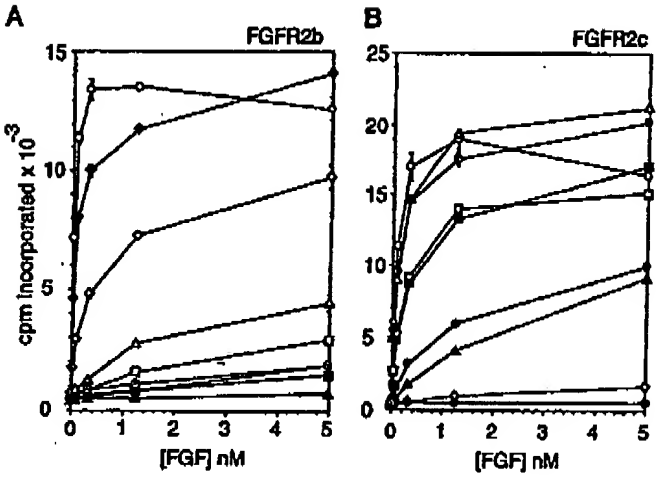

FIG. 3. BaF3 cell mitogenic assays-FGFR 2. *A*, FGFR 2b expressing cells; *B*, FGFR 2c expressing cells. The symbols used to represent FGFs are the same as those shown in Fig. 2.

receptors. Analysis of the FGFR-expressing BaF3 cell lines, by cross-linking of cell surface receptors to labeled FGF 1 or by Western blotting, demonstrates that all cell lines express comparable levels of cell surface receptor (Fig. 1).

FGFR 1 and 2 expressing-BaF3 cells consistently demonstrate a robust mitogenic response to FGF. However, FGFR 3 or FGFR 4 expressing cells respond poorly or not at all to FGF (18, 41).[3] To overcome this diminished signaling capacity, chimeric receptors were engineered to contain the entire extracellular domain of FGFR 3 and FGFR 4 and the tyrosine kinase domain of FGFR 1 (see "Experimental Procedures"). Comparison of the specificity of full-length FGFR 3 expressed in BaF3 cells for FGFs 1, 2, 4, 5, 6, and 7 (18) to chimeric FGFR 3/FGFR 1 (this study) demonstrates complete agreement (see below). We conclude that ligand binding specificity is determined by the extracellular domains of FGFRs and that chimeric receptors mimic their wild-type counterparts with respect to ligand binding specificity.

For FGFRs 1–3 the "long" or three Ig-like domain receptor was used. Unlike for FGFRs 1–3, BaF3 cells expressing the long form of FGFR 4/FGFR 1 chimera demonstrate a significant mitogenic response to anionic polysaccharides, including heparin (42). Therefore, the two Ig-like domain form of FGFR 4 was used. This receptor has a small response to heparin alone but remains responsive to FGF. Preliminary analysis of the long and short forms of FGFRs 1 and 3 demonstrate no significant difference in responsiveness to FGFs, although all nine FGFs have not been examined.[4] The long and short forms of FGFR 4 expressed in BaF3 cells show no significant differences in their ability to bind different FGFs.[5]

Mitogenic Response to FGF—To directly compare the activity of each FGF with a single FGFR, dilutions of FGFs were simultaneously assayed on FGFR-expressing BaF3 cell lines by monitoring [^{3}H]thymidine incorporation into DNA after 36–48 h (Figs. 2–5). All FGFs were bacterially expressed recombinant proteins except for FGF 3, which has not been successfully expressed in bacteria. The FGFs tested were derived from either mouse or human clones. Sequence conservation between mouse and human varies between 80 and 100% amino acid identity (Table II). The higher sequence variability between FGFs 3, 4, and 5 can be accounted for in part by divergence in the region of the sequence encoding the signal peptide (FGF 4 and 5) and the extreme carboxyl terminus (FGF 3 and 5).

[3] D. M. Ornitz, J. Xu, G. Cao, and M. Goldfarb, unpublished data.

[4] A. Chellaiah and D. M. Ornitz, unpublished data.

[5] G. Cao and M. Goldfarb, unpublished data.

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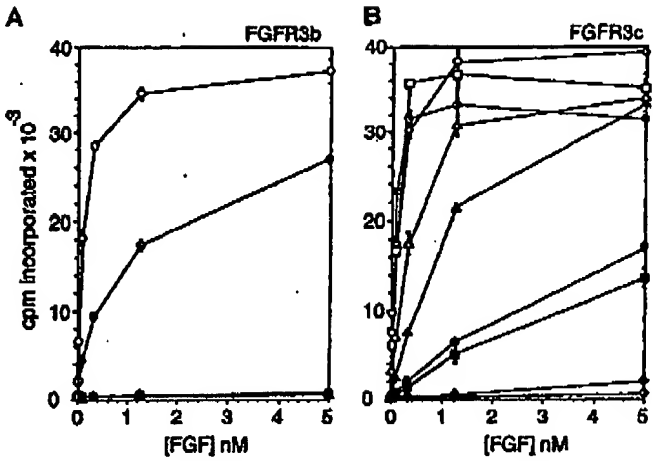

FIG. 4. BaF3 cell mitogenic assays. FGFR 3. *A*, FGFR 3b expressing cells; *B*, FGFR 3c expressing cells. The symbols used to represent FGFs are the same as those shown in Fig. 2.

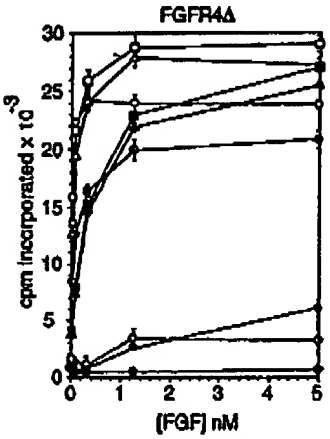

FIG. 5. BaF3 cell mitogenic assays using cells expressing the two Ig-like domain form of FGFR 4. The symbols used to represent FGFs are the same as those shown in Fig. 2.

Concentrations of FGFs ranging from 5 nM to 20 pM were used for these assays. The concentration of heparin, a cofactor required for FGF activity in BaF3 cells (14, 19, 37) was held constant at a concentration of 2 μg/ml. This concentration of heparin is optimal for FGF 1 and FGF 9 (14, 37, 49) and does not demonstrate any inhibitory activity toward FGF 2 (data not shown). Half-maximal activity for the most active FGFs was in the range of 20–300 pM, consistent with the established affinities of the FGFRs for ligand (7, 16). Consistent with previous data in other systems (7, 14–16, 18, 19, 26, 34, 40, 41, 50–55), all forms of FGFRs expressed in BaF3 cells respond to FGF 1.

To assess the relative mitogenic activity of FGFs on individual FGFR splice variants and to make comparisons between different FGF receptors, we normalized the data in Figs. 2–5 to that of FGF 1. To reduce sampling error, the relative mitogenic activity for each ligand was averaged at two different concentrations (312 pM and 1250 pM) (Table III). The concentrations chosen were such that the mitogenic activity was as high as possible, yet the cells were still in near-log phase growth throughout the assay. Recalculating these comparisons at 78 and 312 pM does not significantly change the overall comparison of FGF activity. We interpret a significant mitogenic response as any value greater than 10% of the activity of FGF 1.

The mitogenic activity reported here is in general agreement with the reported receptor binding properties of FGFs. However, there are some notable exceptions. Binding studies on FGFR 4 demonstrate that FGF 2 binds with 10-fold lower affinity than FGF 1 (34), whereas our data indicates that FGF 1 and FGF 2 have similar mitogenic activities (Fig. 5). Similarly, soluble FGFR 3c does not bind FGF 2 (14, 18), whereas BaF3 cells expressing FGFR 3c molecules responds equally well to both FGF 1 and FGF 2 (Ref. 18; Fig. 4*b*). The mitogenic data must therefore be interpreted in the context of the BaF3 cell, the assay conditions used (which include 2 μg/ml heparin) and the absence of endogenous heparan sulfate proteoglycans on the BaF3 cell surface. Different cell types, heparin concentrations, and heparan sulfate proteoglycans may modify the relative specificities reported here.

DISCUSSION

FGFs compose a family of growth factors that play key roles in a variety of developmental events. Many of the FGFs are expressed only in embryonic tissues. However, some of the FGFs continue to be expressed in adult tissues and may be important for maintaining normal tissue homeostasis. FGFs are also involved in mediating a physiological response to injury (3). Diffusion of FGFs from their site of synthesis is limited by their affinity for cell surface and extracellular matrix heparan sulfate (56). Therefore, the tissue-specific expression of FGFs and FGF receptors are critical factors that regulate the activation of the FGF receptor signaling pathway.

Ectopic expression of an FGF ligand or aberrant splicing of an FGF receptor can result in the activation of an autocrine signaling pathway and ensuing uncontrolled cell proliferation. Consistent with this, several of the FGFs are oncogenic when aberrantly expressed in humans or mice (57–59). Additionally, switching in expression from FGFR 2b to FGFR 2c has been implicated in the progression of prostate cancer from a nonmalignant, stromal-dependent, epithelial tumor to an invasive, stromal-independent, undifferentiated tumor (32). This splice form change alters the ligand binding profile of FGFR 2 for both FGF 2 and FGF 7. Coincident with the change in receptor expression, up-regulation of alternative ligands such as FGF 2 has been observed within these epithelial cells (32).

All seven FGFR-expressing BaF3 cell lines respond to FGF 1. This observation is consistent with previously published data (7, 14–16, 18, 19, 26, 40, 50–55). FGF 1 thus appears to be a universal FGFR ligand and may functionally define a core FGF-binding domain. FGF 1 was therefore used as a positive

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TABLE II

Amino acid sequence identity between mouse and human FGFs

Footnotes a and b are the same as in Table I.

FGF	% Amino acid identity	Species used[a]	Ref./source	GenBank accession numbers[b]	
				Human	Mouse
1	96	H	Merck	X51943	M30641
2	94	H	Scios Nova	J04513	M30644
3	80	M	35	X14445	M26284
4	80	H	Genetics Institute	M16838	X14849
5	84	H	38	M37825	M30643
6	93	H	39	X63454	X51552
7	94	H	Amgen	M26295	Z22703
8	100	M	36	U86223	D12482
9	99	M	Pepro Tech, 37	D14838	U93535

[a] M, mouse; H, human.

[b] Accession numbers for sequences used in this analysis.

TABLE III

Relative mitogenic activity of FGFs 1-9

FGF receptor	FGF ligand																	
	FGF-1	± S.D.	FGF-2	± S.D.	FGF-3	± S.D.	FGF-4	± S.D.	FGF-5	± S.D.	FGF-6	± S.D.	FGF-7	± S.D.	FGF-8	± S.D.	FGF-9	± S.D.
1b	100.0	1.6	59.9	1.5	84.4	1.8	15.6	0.1	3.8	0.2	4.6	0.0	6.3	0.3	3.5	0.2	3.5	0.1
1c	100.0	2.0	103.9	2.0	0.3	0.0	102.8	6.4	59.0	1.0	54.9	1.2	0.3	0.0	0.7	0.0	21.1	2.1
2b	100.0	1.7	9.0	0.2	44.8	0.9	14.9	0.3	5.0	0.3	5.4	0.4	80.6	1.1	3.8	0.3	7.3	0.2
2c	100.0	4.5	64.0	3.4	4.2	0.1	94.3	3.9	25.0	0.6	60.7	2.1	2.5	0.1	16.1	0.7	89.2	1.5
3b	100.0	1.8	1.2	0.0	1.5	0.1	1.0	0.1	1.0	0.1	0.9	0.1	1.2	0.1	0.9	0.2	41.5	1.5
3c	100.0	4.5	107.2	1.9	0.6	0.0	69.1	3.8	11.8	0.4	8.8	1.4	1.0	0.1	40.5	0.3	95.6	4.0
4	100.0	3.5	113.4	5.1	5.8	2.2	108.0	3.1	7.0	0.5	79.4	0.3	1.9	1.0	76.1	1.8	75.4	2.9

control and to normalize the mitogenic activity of the other FGFs. Notably, no other FGF ligand could activate all FGFRs.

The expression patterns of FGF receptors 1, 2, and 3 are distinct and analysis of the alternative splicing pattern of these receptors demonstrates that the utilisation of either the b or c exon is dependent upon cell lineage. The b exon appears to be expressed in epithelial tissues while the c exon is expressed in mesenchymal tissues (29, 32, 33). The activity of several of the FGFs toward FGFR isoforms can be divided along these lines. FGF 3 activates the b splice forms of FGFRs 1 and 2, and FGF 7 activates the b splice form of FGFR 2. Expression studies demonstrate that FGF 3 and FGF 7 are expressed in mesenchymal tissues and thus may be paracrine effectors of the overlying epithelia (60–62). In contrast, FGFs 4, 5, 6, and 8 specifically activate c receptor splice forms. Expression studies localize FGF 8 to epithelial tissues and thus FGF 8 may be a paracrine inducer of underlying mesenchyme (36, 63, 64). FGFs 4, 5, and 6 are expressed in both epithelial and mesenchymal lineages and may therefore have either autocrine or paracrine roles (65–68). FGFs 2 and 9 preferentially activate c splice forms; however, FGF 2 shows some activity toward FGFR 1b, and FGF 9 shows some activity toward FGFR 3b. Like FGFs 4, 5, and 6, these FGFs may have both autocrine and paracrine roles.

The diversity in the binding specificity of FGF receptors for FGFs clearly can lead to a large combinatorial set of possible interactions. In addition to the interactions shown in Table III, heterodimers may form between FGF ligands and between FGF receptors. Heterodimers may further increase the repertoire of interactions between FGFs and FGF receptors. Furthermore, the interactions of FGF ligands with heparan sulfate proteoglycans may further affect specificity toward FGF receptors. The concentration of heparin chosen for these studies was such that the activity of FGFs that are known to require relatively high heparin concentrations is optimal. Also, at this concentration, heparin is unlikely to have significant inhibitory activity toward an FGF since the maximum mitogenic activity of individual FGFs is comparable to that of FGF 1 on one or more of the receptor-specific cell lines. With these caveats in mind, this study is nevertheless the first attempt to compare the receptor-specificity of all the FGFs under identical experimental conditions. The important next step will be to determine which ligand-receptor pairs are important in development, in tissue homeostasis and in disease.

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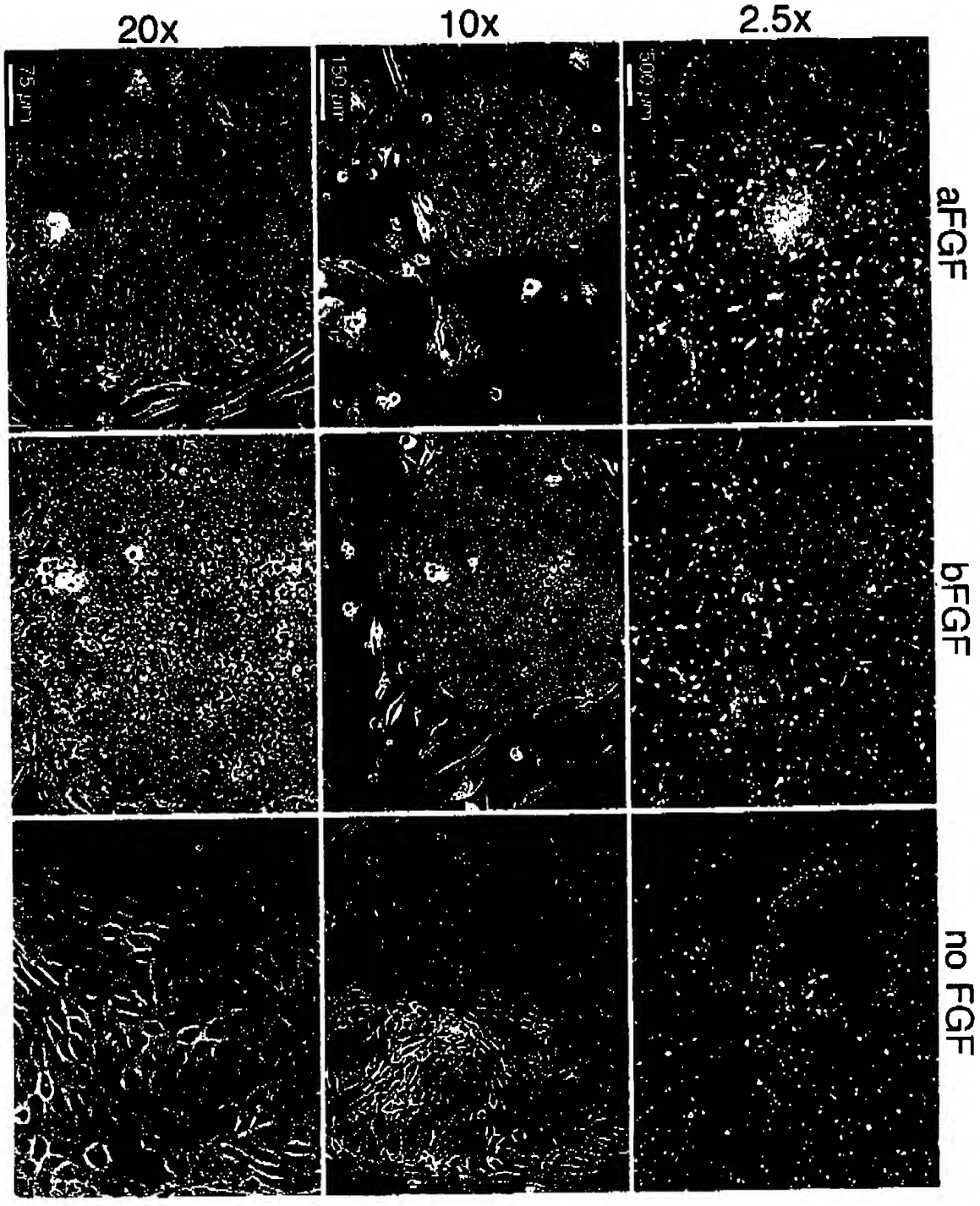

H1 cells plated 4 days prior to imaging in DMEM/F12 based medium supplemented either with aFGF (4ng/ml), bFGF (4ng/ml), or no FGF

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